PDK4 as Marker for PPARdelta Modulation

Peroxisome Proliferator Activated Receptors (PPARs) are members of the nuclear hormone receptor superfamily. The PPARs are ligand-activated transcription factors that regulate gene expression and control multiple metabolic pathways. Three subtypes have been described which are PPARα, PPARδ (also known as PPARβ), and PPARγ. PPARδ is ubiquitously expressed. PPARα is predominantly expressed in the liver, kidney and heart. There are at least two major isoforms of PPARγ. PPARγ1 is expressed in most tissues, and the longer isoform, PPARγ2 is almost exclusively expressed in adipose tissue. The PPARs modulate a variety of physiological responses including regulation of glucose- and lipid-homeostasis and metabolism, energy balance, cell differentiation, inflammation and cardiovascular events.

Approximately half of all patients with coronary artery disease have low concentrations of plasma High Density Lipidprotein Cholesterol (HDL-C). The atheroprotective function of HDL was first highlighted almost 25 years ago and stimulated exploration of the genetic and environmental factors that influence HDL levels. The protective function of HDL comes from its role in a process termed reverse cholesterol transport. HDL mediates the removal of cholesterol from cells in peripheral tissues including those in the atherosclerotic lesions of the arterial wall. HDL then delivers its cholesterol to the liver and sterol-metabolizing organs for conversion to bile and elimination. Data from the Framingham study showed that HDL-C levels are predictive of coronary artery disease risk independently of LDL-C (Low Density Lipidprotein Cholesterol) levels (Gordon et al., *Am. J. Med.* 1977, *62*, 707-714). The estimated age-adjusted prevalence among Americans age 20 and older who have HDL-C of less than 35 mg/dl is 16% (males) and 5.7 % (females). A substantial increase of HDL-C is currently achieved by treatment with niacin in various formulations. However, the substantial side-effects limit the therapeutic potential of this approach.

As many as 90% of the 14 million diagnosed type 2 diabetic patients in the US are overweight or obese, and a high proportion of type 2 diabetic patients have abnormal concentrations of lipoproteins. The prevalence of total cholesterol > 240 mg/dl is 37% in diabetic men and 44% in women. The respective rates for LDL-C > 160 mg/dl are 31%

and 44%, respectively, and for HDL-C < 35 mg/dl 28% and 11%, respectively. Diabetes is a disease in which a patient's ability to control glucose levels in blood is decreased because of partial impairment in response to the action of insulin. Type II diabetes (T2D) is also called non-insulin dependent diabetes mellitus (NIDDM) and afflicts 80-90 % of all diabetic patients in developed countries. In T2D, the pancreatic Islets of Langerhans continue to produce insulin. However, the target organs for insulin action, mainly muscle, liver and adipose tissue, exhibit a profound resistance to insulin stimulation. The body continues to compensate by producing unphysiologically high levels of insulin, which ultimately decreases in later stage of disease, due to exhaustion and failure of pancreatic insulin-producing capacity. Thus T2D is a cardiovascular-metabolic syndrome associated with multiple comorbidities including insulin resistance, dyslipidemia, hypertension, endothelial dysfunction and inflammatory atherosclerosis.

First line treatment for dyslipidemia and diabetes generally involves a low-fat and low-glucose diet, exercise and weight loss. However, compliance can be moderate, and as the disease progresses, treatment of the various metabolic deficiencies becomes necessary with e.g. lipid-modulating agents such as statins and fibrates for dyslipidemia and hypoglycemic drugs, e.g. sulfonylureas or metformin for insulin resistance. A promising new class of drugs has recently been introduced that resensitizes patients to their own insulin (insulin sensitizers), thereby restoring blood glucose and triglyceride levels to normal, and in many cases, obviating or reducing the requirement for exogenous insulin. Pioglitazone (Actos™) and rosiglitazone (Avandia™) belong to the thiazolidinedione (TZD) class of PPARγ-agonists and were the first in their class to be approved for NIDDM in several countries. These compounds, however, suffer from side effects, including rare but severe liver toxicity (as seen with troglitazone). They also increase body weight in patients. Therefore, new, more efficacious drugs with greater safety and lower incidence of side effects are urgently needed. Recent studies provide evidence that agonism of PPARδ would result in compounds with enhanced therapeutic potential, i. e. such compounds should improve the lipid profile, with a superior effect on HDL-C rising compared to current treatments and with additional positive effects on normalization of insulin-levels (Oliver et al; Proc Nat Acad Sci USA 2001; 98: 5306-11). Recent observations also suggest that there is an independent PPARα mediated effect on insulin-sensitization in addition to its well known role in reducing triglycerides (Guerre-Millo et al; J Biol Chem 2000; 275: 16638-16642). Thus, selective PPARδ agonists or PPARδ agonists with additional PPARα activity may show superior therapeutic efficacy without the side-effects such as the weight gain seen with PPARγ agonists.

The present invention relates to a marker for PPARdelta modulation, and methods of diagnosing a disease linked to dysregulation of PPARdelta activity, methods of monitoring the treatment of patients suffering from a disease linked to dysregulation of PPARdelta activity, and methods of identifying compounds which modulate PPARdelta activity.

The gene encoding Pyruvate Dehydrogenase Kinase isoenzyme 4 (PDK4) was identified as direct PPARdelta target gene and this gene may be used as biomarker for PPARdelta. PDK4 is implicated in the metabolic response to fasting.

PDK4 was already described as target gene of PPARalpha (Sugden et al., Diabetes. 2001; 50(12):2729-36). However, the present invention provides that the contribution of PPARdelta on PDK4 gene is at least as large as of PPARalpha in certain tissues and cells. Particularly in muscle cells the PDK4 gene is mainly activated by PPARdelta.

The present invention provides the use of PDK4 as marker for detecting or monitoring PPARdelta modulation. Preferably, PDK4 is used as marker for detecting or monitoring PPARdelta modulation in muscle cells. PDK 4 may also be used for determining toxicity in muscle cells. A muscle cell may be a skeletal muscle cell or a heart muscle cell.

The term "modulation" as used herein relates to an activation or inhibition of the transcriptional activity of PPARdelta. Thus, PDK4 can serve as marker for modulation of PPARdelta activity.

The term "marker" as used herein refers to nucleic acid or polypeptide.

The present invention also pertains to PDK4 as marker for diagnosing a disease involving dysregulation of PPARdelta activity such as for example dyslipidemia, obesity or insulin resistance. Thus, PDK4 can serve as marker detecting for the modulation of PPARdelta activity.

The present invention further provides a method of detecting or monitoring the activity of PPARdelta in a host comprising quantifying the expression level of PDK4 mRNA. A representative cDNA of mouse PDK4 is shown in SEQ. ID NO: 1.

In one embodiment of the method hereinbefore described the mRNA expression level of PDK4 is determined relative to a control.

A host may be an animal, tissue, cells or any other biological system that is capable of RNA transcription including in vitro transcription. Preferably, the animal is a non-human animal. The control may be the level of mRNA expression of PDK4 in a different host.

Furthermore, the present invention provides a method of determining whether a test compound modulates PPARdelta activity in a host comprising

a) exposing the host to the test compound and

b) quantifying the mRNA expression level of PDK4.

In one embodiment of the method hereinbefore described the mRNA expression level of PDK4 is determined relative to a control.

A host may be an animal, tissue, cells or any other biological system that is capable of RNA transcription including in vitro transcription. Preferably, the animal is a non-human animal. The control is the level of the mRNA expression of PDK4 in an untreated host, which may be the said host before the treatment or a different host, and/or the said host after an appropriate period of treatment for normalization to pretreatment levels. In a preferred embodiment of the method hereinbefore described, the compound that modulates PPARdelta activity is either an antagonist or an agonist.

The present invention also provides a method for monitoring treatment of patients suffering from a disease associated with dysregulation of PPARdelta activity such as for example dyslipidemia, obesity or insulin resistance, comprising the steps of:

a) purifying mRNA from muscle cells isolated from patients treated with a modulator of PPARdelta activity and

b) measuring the mRNA expression of PDK4.

In one embodiment of the method hereinbefore described the mRNA expression level of PDK4 is determined relative to a control.

A host may be an animal, tissue, cells or any other biological system that is capable of RNA transcription including in vitro transcription. Preferably, the animal is a non-human animal. The control is the level of the mRNA expression of PDK4 in an untreated host, which may be the said host before the treatment or a different host, and/or the said host after an appropriate period of treatment for normalization to pretreatment levels.

The present invention also pertains to compounds identified by the methods hereinbefore described, and to the use of compounds identified by a method hereinbefore described for the preparation of a medicament for the treatment of a disease involving dysregulation of PPARdelta activity such as for example dyslipidemia, obesity or insulin resistance.

Several methods for measuring expression levels of PDK4 mRNA can be used. Methods such as Northern Blotting, and quantitation of the bands by densitometry are well known in the art and may be used, although they may not be sufficiently accurate. Other methods include the use of genechips, microarray analysis, dot blotting or different quantitative PCR methodologies. Preferably, Taqman or real time quantitative PCR is used. Any part of the transcribed sequence of PDK4 may be used as probe. In a preferred embodiment, mRNA expression levels of PDK4 are determined by real-time quantitative PCR using the forward primer and reverse primer listed in table 2 (SEQ. ID NOs: 4 and 5). Preferably, the mRNA expression level of PDK4 in muscle cells is quantified.

The present invention further provides a method of detecting or monitoring the activity of PPARdelta in a host comprising quantifying the expression level of PDK4 protein. SEQ. ID NO: 3 shows the protein sequence of mouse PDK4.

In one embodiment of the method hereinbefore described the protein expression level of PDK4 is determined relative to a control.

A host may be an animal, tissue, cells or any other biological system that is capable of protein translation including in vitro translation. Preferably, the animal is a non-human animal. The control is the level of the protein expression of PDK4 in a different host.

Further to this the present invention provides a method of determining whether a test compound modulates PPARdelta activity in a host comprising

a) exposing the host to the test compound and

b) quantifying the protein expression level of PDK4.

In one embodiment of the method hereinbefore the protein expression level of PDK4 is determined relative to a control.

A host may be an animal, tissue, cells or any other biological system that is capable of protein translation including in vitro translation. Preferably, the animal is a non-human animal. The control is the level of the protein expression of PDK4 in an untreated host, which may be the said host before the treatment or a different host, and/or the said host after an appropriate period of treatment for normalization to pretreatment levels. The host may be treated with a carrier. The carrier may be a solvent in which the compound is dissolved or resuspended. In a preferred embodiment of the method hereinbefore described, the compound that modulates PPARdelta activity is either an antagonist or an agonist.

Furthermore, the present invention also provides a method for monitoring treatment of patients suffering from a disease associated with dysregulation of PPARdelta activity such as for example dyslipidemia, obesity or insulin resistance, comprising the steps of:

a) purifying protein from muscle cells isolated from patients treated with a modulator of PPARdelta activity and

b) measuring the protein expression of PDK4.

In one embodiment of the method hereinbefore the protein expression level of PDK4 is determined relative to a control.

A host may be an animal, tissue, cells or any other biological system that is capable of protein translation including in vitro translation. Preferably, the animal is a non-human animal. The control is the level of the protein expression of PDK4 in an untreated host, which may be the said host before the treatment or a different host, and/or the said host after an appropriate period of treatment for normalization to pretreatment levels.

Several methods for measuring expression levels of PDK4 protein can be used. These methods include but are not limited to two-dimensional gel separation, mass-spectrometry, antibody binding techniques (ELISA, western blot) and immunoprecipitation. Preferably, the protein expression level of PDK4 in muscle cells is quantified.

The present invention also pertains to compounds identified by the methods hereinbefore described, and to the use of compounds identified by a method hereinbefore described for the preparation of a medicament for the treatment of a disease involving dysregulation of PPARdelta activity such as for example dyslipidemia, obesity or insulin resistance.

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

Figures

Figure 1: Graphical representation from PDK4 gene expression fold changes induced by PPAR ligands in Table 1 (A 300µM, B 100nM, C 500nM) in C2C12 mouse muscle cells measured by Affymetrix micro-arrays. PDK4: Pyruvat Dehydrogenase Kinase isoenzyme 4 uced Adipose Factor [marker for PPARdelta activity], A: 2-[4-(4-Chlorobenzoyl)-phenoxy]-2-methylpropanoic acid (fenofibric acid) [PPARalpha agonist], B: {2-Methyl-4-[4-Methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulfanyl]-phenoxy}-acetic acid [PPARdelta-alpha co-agonist], C: [rac]-(4-{Cyclopentyl-[4-methyl-2-(4-trifluoromethyl-penyl)-thiazol-5-yl]-methylsulfanyl}-naphtalen-1-yloxy}-acetic [PPAR delta agonist].

Figure 2: Graphical representation from PDK4 gene expression fold changes induced by PPAR ligands in Table 3 (A 300µM, B 100nM, C 500nM, D 500nM) in C2C12 mouse muscle cells measured by qPCR. PDK4: Pyruvat Dehydrogenase Kinase isoenzyme 4 [marker for PPARdelta activity], A: 2-[4-(4-Chlorobenzoyl)-phenoxy]-2-methylpropanoic acid (fenofibric acid) [PPARalpha agonist], B: {2-Methyl-4-[4-Methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulfanyl]-phenoxy}-acetic acid [PPARdelta-alpha co-agonist], C: [rac]-(4-{Cyclopentyl-[4-methyl-2-(4-trifluoromethyl-penyl)-thiazol-5-yl]-methylsulfanyl}-naphtalen-1-yloxy}-acetic [PPARdelta agonist], D: (2-Methyl-4-{Methyl-[4-methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethyl]-amino}-phenoxy)-acetic acid [PPAR delta-alpha co-agonist].

Examples

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

Example 1: Microarray experiment

Cell culture: C2C12 cells (ATCC: CRL1772)

Cells were cultured in DMEM (5 mM glucose) supplemented with 10 % heat-inactivated fetal calf serum (FCS). Complete medium was filtered through 0.2 µm pores before use. Differentiation was performed in 6 well dishes by serum depleting the medium (DMEM High Glucose, 2% FCS) when cells reach confluency. Cell cultures were then incubated for 5 days at 37°C in a 90%-humidified and 95%-air-5%-CO_2 sterile atmosphere to allow differentiation.

PPAR Ligands were dissolved in 100% DMSO and added to medium to adjust 0.1% (v/v) DMSO final concentration and cells were incubated with ligands for 20 hours under normal growth conditions. Three replicates were done for each condition.

RNA extraction

RNA extraction procedure has been performed with Qiagen QIAschredder and RNeasy kits.

Approximately $1*10^6$ pelleted cells per sample were resuspended in 350 µl guanidine thiocyanate containing RLT. Samples were loaded on a QIAshredder-column and centrifuged at full speed for 2 minutes to homogenize the lysates. After adding 350 µl ethanol to the flow-through for better binding conditions, samples were applied to RNeasy spin. For the first wash step 350 µl RW1 buffer were pipeted into the column. Then 10 µl DNase solution gently mixed with 70 µl RDD buffer were applied onto the column and incubated for 15 minutes at room temperature to remove genomic DNA. DNase was washed out with 350 µl RW1 buffer. Two purifying wash steps followed by adding 500 µl RPE buffer. RNA was eluted in water and stored at - 70°C.

cDNA synthesis

cDNA was synthesized using the cDNA synthesis system kit supplied by Roche Diagnostics and following the manufacturer's manual. During the whole synthesis it was worked on ice.

For the first strand synthesis, 2 µl oligo d(T7)T_{24} primer (5'GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(T)_{24}$VN 3', 200 pmol/µl) and redist water were added to up to 20 µg RNA for a final reaction volume of 21 µl. After incubation at 70°C for 10 minutes in a thermal cycler and hybridization of the primers to the RNA, 2 µl AMV reverse transcriptase and 4 µl dNTP-mix (10 mM) were added. Supplementary 8 µl RT-buffer, 4 µl DTT (0.1 mM) and 1 µl RNase inhibitor were pipeted into the mixture. Following incubation at 42°C dured 60 minutes.

Second strand synthesis reagents were added directly into the first strand reaction tube. The second strand enzyme blend, of which 6.5 µl were applied to the mixture, contains RNase for inserting nicks into RNA of the DNA/RNA hybrid. This provides 3'OH-primers for DNA polymerase I which is also present in the 2nd strand enzyme cocktail as well as E.coli ligase too. Additionally 1.5 µl dNTP-mixture, 30 µl 2nd strand buffer and 72 µl redist water were added and mixed gently. The reaction mixture was incubated for 2 hours at 16°C. 20 µl added T4 polymerase ensured that the termini of the cDNA were blunt after 5 minutes incubation at 16°C. Then samples were treated with RNase to remove RNA template (30 minutes at 37°C) and proteinase K (30 minutes, 37°C).

Purification of cDNA

Purification using the QIAquick PCR purification kit was supplied by Quiagen.

850 µl PB buffer were added to the cDNA reaction tube and mixed. Sample were applied to the QIAquick column and centrifuged for 30 seconds at 13000 rpm. 750 µl PE buffer were added to the column to wash cDNA. An additional centrifugation step at maximum speed followed which completely dried the column. To elute DNA, 50 – 80 µl EB buffer (10 mM Tris-Cl, pH: 8.5) were added to the center of the membrane and the sample was centrifuged for one minute at 13000 rpm.

The cDNA quality was checked by agarose gel electrophoresis and ethidium bromide staining.

Smears from 100 to >10000 bp appeared. The quantity and purity of the synthesized cDNA was determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer.

The concentration was calculated as follows: $c[\mu g/ml] = A_{260} * 50 * D$, (50: dsDNA specific factor, D: dilution factor). cDNA has a A_{260}/A_{280}-ratio of 1.8-2.1

In vitro Transcription (IVT)

This step was performed using MEGAscript T7 kit from Ambion.

5 µg cDNA from each samples were used as template and mixed with ATP, GTP, CTP, UTP (Ambion) and biotinylated CTP (Bio-11-CTP, ENZO) and UTP (Bio-15-UTP, ENZO). RT was performed by T7 enzyme mix for 4 hours at 37°C. Transcription products were purified with RNeasy kit from Qiagen following the same procedure as for RNA extraction, without any DNAse treatment of the samples.

Fragmentation of IVT products

15 µg RNA from IVT were fragmented in 200 mM Tris-Acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc for 35 minutes at 95°C in a small volume (20 to 30 µl).

Chip Handling (cf DNA Micro-array Protocols V3-200.1. M Wilhelm-Seiler, U Certa)

A pre-treatment solution was first applied on Genechip (6.25 µl Acetylated BSA (20 µg/µl), 12.5 µl salmon sperm DNA (10 µg/µl), 125 µl 2x MES Hyb buffer, 106.25 µl H_2O). Genechip were incubated for 15 min at 40°C with a rotation of 60 rpm in a rotisserie.

Samples were prepared for hybridization as follows: mix 15 µg fragmented RNA with 2.5 µl control stock mix which contains BioB, BioC, BioD, Cre (internal references),

2.5 µl salmon sperm DNA (10 µg/µl), 6.25 µl Acetylated BSA (20 µg/µl), 125 µl 2x MES-Hyb buffer, 91.25 µl H_2O. Microarrays were then incubated overnight at 45°C with rotation (60 rpm) in a rotisserie.

Samples were removed and stored at -20°C. Microarrays were washed with 6 SSPE for 5 minutes in the fluidics. Then 230 µl MES-Wash buffer were applied to the chip which was incubated at 45°C for 30 minutes, 60 rpm.

Staining was performed by incubating chips with the following solution for 15 minutes: 125 µl 2x Stain Buffer, 91.25 Acetylated BSA, 2.5 µl Streptavidin (1 mg/ml).

Staining solution was removed, micro-arrays washed with 6x SSPE for 5minutes in the fluidics Station, and treated with the amplification solution: 125 µl 2x Stain Buffer, 99 µl H_2O, 1 µl Biotinylated-anti-Streptavidin (500 µg/ml), 25 µl Acetylated BSA (20 µg/µl). Incubation for 30 minutes at 40°C with 60 rpm in the rotisserie. After washing I the fluidics Station with 6x SSPE, micro-arrays were incubated with phycoerytrin solution (125 µl 2x Stain buffer, 91.25 µl H_2O, 31.25 µl acetylated BSA, 2.5 µl Phycoerytrin (1 mg/ml)) at 40°C, with 60 rpm.

Solutions:

12x MES stock :	70.4 g MES free acid monohydrate, 193.3 g MES sodium salt, adjust pH to 6.6, ad 1000 ml with tridest H_2O
2x MES-Hyb Buffer :	8.3 ml 12x MES stock, 17.7 ml 5M NaCl, 4 ml 0.5M EDTA, 0.1 ml 10% Tween 20, 19.9 ml tridest H_2O
MES-Wash Buffer :	83.3 ml 12x MES stock, 5.2 ml 5M NaCl, 1 ml 10% Tween 20, ad 1000 ml with tridest H_2O
6x SSPE Tween :	300 ml 20x SSPE, 1 ml 10% Tween 20, ad 1000ml tridest H_2O
2x Stain Buffer :	41.7 ml 12x MES stock, 92.5 ml 5M NaCl, 2.5 ml 10% Tween 20, 113.3 ml tridest H_2O

Scanning is performed with Affymetrix scanner and results were provided on Gene Chip software from Affymetrix.

Chip Data Analysis

Data were analyzed using Race A Sofware (Bioinformatic Roche). Triplicates were used for each condition and comparisons were calculated for each condition referring to the control (untreated cell samples). Several criteria have been taken into account for the analysis of each gene: pValue, Call average, Sum average difference, Change Factor and dispersion in each condition to get a list of the most statistically relevant genes that were modulated by the studied compounds.

Results

A novel selective PPAR delta biomarker gene was identified from Affymetrix micro-array experiments (see Fig.1):. PDK4 (Pyruvate Dehydrogenase Isoenzyme 4) (SEQ. ID NO: 2). This marker gene allows to differentiate PPAR alpha from PPAR delta activities.

The tested ligands were a PPARalpha agonist (A: 2-[4-(4-Chlorobenzoyl)-phenoxy]-2-methylpropanoic acid (fenofibric acid)), a PPARdelta agonist (C: [rac]-(4-{Cyclopentyl-[4-methyl-2-(4-trifluoromethyl-penyl)-thiazol-5-yl]-methylsulfanyl}-naphtalen-1-yloxy}-acetic) and a PPARdelta-alpha co-agonist (B: {2-Methyl-4-[4-Methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulfanyl]-phenoxy}-acetic acid).

As shown in Table 1 and Fig. 1 the response of the identified marker gene PDK4 elicit by the PPARdelta agonist (C) and PPARdelta-alpha co-agonist (B) is larger than the response elicited by the PPARalpha specific ligand (A).

	Symbol	Control	A	B	C
pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	1.00	3.30	5.95	6.06

Table 1: PDK4 gene expression fold changes in C2C12 mouse muscle cells induced by PPAR ligands: A 300 µM, B 100 nM, C 500 nM measured by Affymetrix micro-arrays. (A: 2-[4-(4-Chlorobenzoyl)-phenoxy]-2-methylpropanoic acid (fenofibric acid) is a

PPARalpha agonist, B: {2-Methyl-4-[4-Methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulfanyl]-phenoxy}-acetic acid is a PPARdelta-alpha co-agonist, C: [rac]-(4-{Cyclopentyl-[4-methyl-2-(4-trifluoromethyl-penyl)-thiazol-5-yl]-methylsulfanyl}-naphtalen-1-yloxy}-acetic is a PPARdelta agonist)

Example 2: quantitative PCR

The culturing of C2C12 mouse muscle cells, RNA extraction, cDNA synthesis and purifications were performed as described in Example 1.

PCR Primer Design

Primer pairs were designed with the software Primer express 1.0 from PE Applied Biosystems based on mRNA/cDNA sequences from Medline. Criteria for each primer were length between 19 to 21 nucleotides, melting temperature of 60 °C +/- 1, G/C content range from 40 to 60% and avoidance of secondary structures as hairpins or primer dimers. Additionally the length of the amplicon was controlled as shorter amplicons amplify more efficiently than longer ones and are more tolerant of reaction conditions (usually ~100 bp). Primers were dissolved in DEPC treated water (Ambion) to a 100 µM concentrated solution and stored at –20 °C.

GENE	ACC	OLIGO NAME	FORWARD PRIMER	SEQ. ID:	OLIGO NAME	REVERSE PRIMER	SEQ. ID:
S12	Y11682	mS12_98F	TGAACCAGATGCACCGCTTAG	6	mS12_416R	TTCTTCTTTTGCACGTGGCC	7
PDK4	BC026134	mPDK4-1_1632F	TTCAGTGTTTCCCCGCCAT	4	mPDK4-1_1978R	TCCCAACATGCACAATCCTTG	5

Table 2: Forward and reverse primers of gene S12 (internal reference) and PDK4.

qPCR using Corbett RG3000 and ABI 7000

RT-PCR was performed with the Quantitech SYBR Green kit from Qiagen according to the manufacturer's manual. Each component were added to 96 well plate: 25 µl master mix, 0.16 µl of each primer (100 µM stock solution), 22.7 µl H_2O and 2 µl cDNA.

The fluorescent dye SYBR green I binds the minor groove of double stranded DNA and allows measurement of DNA quantity at every cycle. The program consists of three different steps: initial denaturation of template and heat-activation of the Taq DNA

polymerase (95°C, 15 min), cycling (95°C denaturation, 60 °C primer-template annealing, 72°C elongation, fluorescence aquisition), and final melting (melting curve 55 to 95°C, measurements in 0.5 °C steps). The melting curve allows the discrimination between specific and non-specific amplification products (primer dimers).

Relative quantification using real-time PCR

The relative quantification is based on the relative expression of the target gene versus a reference gene. The concept of threshold fluorescence, defined as the point where fluorescence rises above the background fluorescence enables accurate and reproducible quantification of gene expression. The cycle number at which threshold fluorescence is reached, is called ct value. A linear relation between ct value and the log of the number of initial molecules is given during the exponential phase of the amplification reaction. Therefore, quantification is reliable at that stage and will not be affected by any limiting effects of the components. The relative ct value of a target gene compared to the ct value of an internal reference in the control or untreated sample is expressed as Δct.control = $ct_{\text{reference of control}} - ct_{\text{target of control}}$ and in a treated, unknown sample as Δct.sample= $ct_{\text{reference of sample}} - ct_{\text{target of sample}}$.

The theoretical PCR reaction is expressed through the formula $N = N_0 * 2^n$ (N: number of amplified molecules, N_0: initial number of molecules, n: number of amplification cycles) where the base 2 represents the optimal efficiency (one doubling every cycle). This equation allows to illustrate differential expression in mRNA copy number instead of ct values: the exponential value with the theoretical efficiency, $2^{\Delta ct}$, is taken.

As internal reference for quantitative studies the ribosomal protein S12 gene expression was used. It is expressed constitutively and at the same level in all samples. Setting of fluorescence threshold is determined by the light cycler software. The obtained ct values (duplicates for one sample, two samples per condition) were exported into Microsoft Excel and analyzed as described above.

Results

The novel selective PPAR delta biomarker gene (SEQ. ID. NO: 2) identified from Affymetrix micro-array experiments (see Fig.1) was confirmed by qPCR (quantitative polymerase chain reaction).

The tested ligands were a PPARalpha agonist (A: 2-[4-(4-Chlorobenzoyl)-phenoxy]-2-methylpropanoic acid (fenofibric acid)), a PPARdelta agonist (C: rac]-(4-{Cyclopentyl-[4-methyl-2-(4-trifluoromethyl-penyl)-thiazol-5-yl]-methylsulfanyl}-naphtalen-1-yloxy}-acetic) and two PPARdelta-alpha co-agonists (B: {2-Methyl-4-[4-Methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulfanyl]-phenoxy}-acetic acid and D: (2-Methyl-4-{Methyl-[4-methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethyl]- amino}-phenoxy)-acetic acid).

As shown in Table 3 and Fig. 2 the response of the marker gene PDK4 elicited by the PPARdelta agonist (C) and PPARdelta-alpha co-agonists (B and D) is larger than the response elicited by the PPARalpha specific ligand (A).

	Symbol	Control	A	B	C	D
pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	1	1.77	11.06	4.99	8.63

Table 3: PDK4 gene expression fold changes in C2C12 mouse muscle cells induced by PPAR ligands in C2C12: A 300 μM, B 100 nM, C 500 nM, D 500nM as measured by qPCR. (A: 2-[4-(4-Chlorobenzoyl)-phenoxy]-2-methylpropanoic acid (fenofibric acid) is a PPARalpha agonist, B: {2-Methyl-4-[4-Methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulfanyl]-phenoxy}-acetic acid is a PPARdelta co-agonist, C: [rac]-(4-{Cyclopentyl-[4-methyl-2-(4-trifluoromethyl-penyl)-thiazol-5-yl]-methylsulfanyl}-naphtalen-1-yloxy}-acetic is a PPARdelta agonist, D: (2-Methyl-4-{Methyl-[4-methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethyl]- amino}-phenoxy)-acetic acid is a PPARdelta-alpha co-agonist)